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Authors
Dziennis, S
Van Etten, R A
Pahl, H L
et al.

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RAPID COMMUNICATION

The CD11b Promoter Directs High-Level Expression of Reporter Genes in Macrophages in Transgenic Mice

By Suzan Dziennis, Richard A. Van Etten, Heike L. Pahl, Dale L. Morris, Thomas L. Rothstein, Consuelo M. Blosch, Roger M. Perlmutter, and Daniel G. Tenen

CD11b is the α chain of the Mac-1 integrin and is preferentially expressed in myeloid cells (neutrophils, monocytes, and macrophages). We have previously shown that the CD11b promoter directs cell-type–specific expression in myeloid lines using transient transfection assays. To confirm that these promoter sequences contain the proper regulatory elements for correct myeloid expression of CD11b in vivo, we have used the −1.7-kb human CD11b promoter to direct reporter gene expression in transgenic mice. Stable founder lines were generated with two different reporter genes, a Thy 1.1 surface marker and the *Escherichia coli* lacZ (β-galactosidase) gene. Analysis of founders generated with each reporter demonstrated that the CD11b promoter was capable of driving high levels of transgene expression in murine macrophages for the lifetime of the animals. Similar to the endogenous gene, transgene expression was preferentially found in mature monocytes, macrophages, and neutrophils and not in myeloid precursors. These experiments indicate that the −1.7 CD11b promoter contains the regulatory elements sufficient for high-level macrophage expression. This promoter should be useful for targeting heterologous gene expression to mature myeloid cells.
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DURING HEMATOPOIESIS, immature myeloid progenitor cells undergo a program of differentiation that results in the production of mature neutrophils and monocytes; the latter subsequently undergo further maturation into macrophages.[1] Normal differentiation is accompanied by distinct changes in gene expression. The importance of understanding this process is underscored by the finding that most adult acute leukemia is characterized by a block in the differentiation program.[2]

One antigen upregulated during myeloid differentiation is the α subunit of the Mac-1 leukocyte integrin heterodimer, CD11b, which serves as an important adhesion molecule mediating many of the functions of these phagocytic cells.[3] CD11b is expressed at low or undetectable levels in myeloid precursors, and its expression increases steadily during myeloid differentiation, reaching highest levels in mature neutrophils, monocytes, and macrophages.[4-6] CD11b surface expression parallels mRNA levels, which appear to be largely regulated at a transcriptional level.[6,7] Although CD11b is preferentially expressed on myeloid cells, surface expression has also been detected on minor subsets of T and B cells, including the $CD5^+$ ($Ly1^+$) (B-1) B-cell subset,[8-11] but not on other hematopoietic or nonhematopoietic tissues.

Analysis of the CD11b promoter in cell lines has led to important insights into myeloid gene regulation.[7,12] These studies have identified factors important for CD11b expression as well as for other myeloid genes. For example, PU.1 (Spi-1), which is critical for CD11b promoter activity,[7,13] also regulates a growing number of myeloid-specific genes.[14-18] In addition, its function is essential for myeloid colony formation.[19] These studies indicated that the −1.7-kb CD11b promoter directed myeloid-specific reporter gene activity in transient transfection of leukemic cell lines. However, whether these data accurately reflect the regulation of the CD11b promoter in normal cells has not been determined. We have therefore analyzed the expression of CD11b promoter constructs in transgenic mice to determine the *cis*-acting DNA sequences that direct myeloid-specific as well as high-level long-term expression in vivo, studies that facilitate the expression of heterologous genes in myeloid cells in vivo. These studies show that the −1.7-kb CD11b promoter can direct high-level expression of heterologous reporter genes in murine macrophages.

MATERIALS AND METHODS

Construction of the CD11b/Thy1.1 and CD11b/β-gal transgenes. The CD11b promoter fragment used in these studies extended from bp −1704 to +83 and therefore included 83 bp of the 5′ untranslated region extending up to the ATG start codon (Fig 1A).[7,20] This genomic fragment was subcloned from a CD11b genomic λ clone[20] into pGEM3zf−, isolated by restriction endonuclease digestion with *Hin*dIII and *Sma* I, and subsequently ligated into the *Hin*dIII/*Eco*RV sites of pBluescript KS (pKS). A 2.1-kb *Bam*HI/*Not* I fragment containing the human growth hormone (HGH) gene was inserted into the *Bam*HI/*Not* I sites of pKS to create pB202. Finally, a 544-bp *Bam*HI fragment containing the murine Thy1.1 cDNA was inserted into the *Bam*HI site of pB202 to produce pB203, containing the −1.7-kb CD11b promoter 5′ of the Thy1.1 cDNA and hGH gene in pKS (Fig 1B). To synthesize a CD11b reporter construct using an alternative reporter, the *Hin*dIII/*Sma* I CD11b fragment was blunt end ligated into the *Hin*dIII site of pSDKlacZpA[21] to form plasmid T133, containing the −1.7-kb CD11b promoter 5′ of the lacZ gene (Fig 1C).

From the Hematology/Oncology Division, Department of Medicine, Beth Israel Hospital, and Center for Blood Research, Harvard Medical School, Boston, MA; the Departments of Medicine and Microbiology, Boston University Medical Center, Boston, MA; and Howard Hughes Medical Institute and Departments of Immunology, Biochemistry, and Medicine, University of Washington, Seattle, WA.
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Address reprint requests to Daniel G. Tenen, MD, Research East 219, Beth Israel Hospital, 330 Brookline Ave, Boston, MA 02215.
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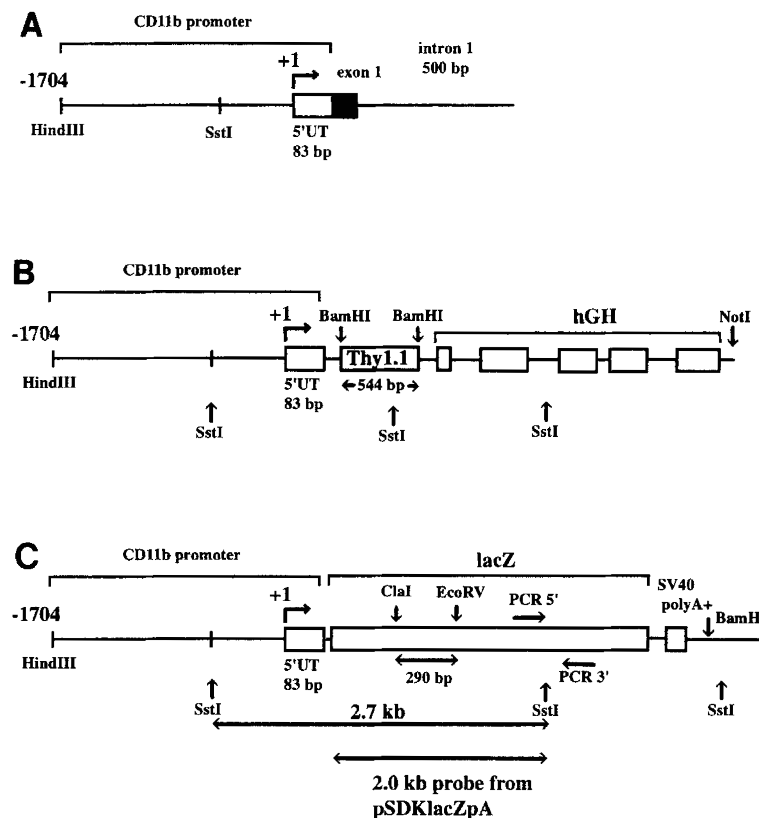

Fig 1. CD11b transgenic constructs used in these studies. (A) Map of the human CD11b 5′ region. Pictured is the structure of the human CD11b gene extending from the *HindIII* site at bp −1,704 and extending into the first intron.[20] Shown is the *Sst* I site at bp −663, the transcription start site at bp +1, and exon 1, which includes 83 bp of 5′ untranslated region and the first 28 bp of the coding region. Intron 1 is 500 bp in length. The CD11b promoter fragment used in these studies is actually 1,787 bp, extending from the *HindIII* site at bp −1,704 to bp +83 of the 5′ untranslated region. The maps in Fig 1 are not drawn to scale. (B) CD11b/Thy1.1 reporter transgenic construct. The 1,787-bp CD11b promoter construct was ligated 5′ of a 544-bp Thy1.1 cDNA. Distal to the Thy1.1 cDNA is a 2.1-kb *BamHI/Not* I fragment containing the human growth hormone gene, which serves to provide splice donor/acceptors sites to enhance transgene expression[44] as well as a polyadenylation signal. Exons in the HGH gene are presented as boxes. DNA was isolated after digestion with *HindIII* and *Not* I before injection into embryos for production of the transgenic line. The probe used for Southern blot analysis of founder copy number was the 544-bp *BamHI* fragment containing the Thy1.1 cDNA. (C) β-gal reporter. The 1,787-bp CD11b promoter was blunt end ligated into the plasmid pSDKlacZpA, which contains the lacZ gene encoding β-galactosidase.[21] At the 3′ end of the β-gal coding region is an SV40-derived splice donor/acceptor and polyadenylation site. Shown are the locations of the single *BamHI* site in the construct (*BamHI* was used to linearize the plasmid pSDKlacZpA as a standard on Southern blots), the other *Sst* I sites in the transgenic construct, as well as the location of the 2.0-kb *Sst* I fragment from pSDKlacZpA used as a probe. Also shown are the *Cla* I and *EcoRV* sites used to generate the probe used in RNAse protection assays, as well as the location of the PCR primers (PCR 5′ and PCR 3′) used for screening founder animals. The *HindIII/BamHI* fragment was isolated and used to generate transgenic founders.

Production of transgenic mice. The CD11b/Thy1.1 and CD11b/β-gal transgenes were isolated away from vector sequences by digestion with *Hind*III/*Not* I and *Hind*III/*Bam*HI, respectively, followed by agarose gel electrophoresis. DNA fragments were then purified by electroelution or glass bead extraction. Transgenic mice were generated by microinjection into male pronuclei of single-cell embryos and reimplantation into pseudopregnant recipient females. CD11b/Thy1.1 transgenics were established in C57BL mice, which are homozygous for the Thy1.2 allele, whereas the CD11b/β-gal mice were established in the FVB line, which is homozygous for the Thy1.1 allele.

In the case of the CD11b/β-gal construct, founders were identified first by polymerase chain reaction (PCR) of tail snip DNA, using as primers 5′-ATACGCCGAACGATCGCCAGTTCT-3′ and 5′-CACTACGCGTACTGTGAGCCAGAG-3′, representing bp 1781-1804 and 2103-2080 of the lacZ gene, respectively.[22] PCR-positive CD11b/β-gal mice were confirmed and CD11b/Thy1.1 mice were identified by Southern blot analysis as described below.

Assessment of transgene copy number by Southern blot analysis. Confirmation of integration of the transgene and assessment of copy number was performed by Southern blot. Genomic DNA was prepared from tails of mice by digestion overnight with 100 μg/mL of proteinase K in 10 mmol/L Tris, pH 8.0, 100 mmol/L NaCl, 10 mmol/L EDTA, and 0.5% sodium dodecyl sulfate (SDS). The DNA was then extracted with a mixture of 50:48:2 phenol:chloroform:isoamyl alcohol and ethanol-precipitated. Ten micrograms of DNA was then cut to completion overnight with the restriction enzymes as described in the legend to Fig 2, separated on 1% agarose gels, and capillary blotted to nylon membranes (Biotrans; ICN, Irvine, CA). Plasmid DNA was mixed with 10 μg of similarly prepared genomic DNA from nontransgenic littermates and run in adjacent lanes to establish standards for copy number. Membranes were baked

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at 80°C, UV cross-linked for 5 minutes under tissue culture hood UV lights, and hybridized to randomly primed cDNA probes labeled with ^{32}P-dCTP in 7% SDS, 500 mmol/L $NaPO_4$, 1% bovine serum albumin (BSA; Pentax Fraction V; Sigma, St Louis, MO). Blots were then washed at 65°C in 150 mmol/L $NaPO_4$, pH 7.2, 0.1% SDS, followed by a second wash in 30 mmol/L $NaPO_4$, pH 7.2, 0.1% SDS, and subjected to autoradiography at −80°C with an intensifying screen for the times indicated in the figure legends. Intensity of bands was quantitated using Imagequant phosphorimager software (Molecular Dynamics, Sunnydale, CA).

Isolation of leukocytes and peritoneal cells in transgenic mice. Peripheral blood leukocytes were isolated by separation in Ficoll-Hypaque gradients, hypotonic lysis in 0.15 mmol/L NH_4Cl, 1 mmol/L $KHCO_3$, 0.1 mmol/L EDTA followed by washing in phosphate-buffered saline (PBS). Peritoneal cells were isolated by peritoneal lavage with 20 mL PBS at times indicated after the intraperitoneal administration of 1 mL of a 10% solution of thioglycollate (DIFCO, Detroit, MI). Bone marrow was flushed from femurs and tibias with PBS. Assessment of cell morphology of bone marrow and peritoneal cells was performed by Wright-Giemsa staining of slides after cytocentrifugation. CD5$^+$ (B-1) B cells were isolated from peritoneal cells of non−thioglycollate-treated animals as described,[23] using a combination of T-cell complement-mediated lysis, depletion of macrophages by adherence, removal of residual macrophages and other nonlymphoid cells by sedimentation on discontinuous Percoll gradients, and removal of CD5$^-$ (B-2) B cells with antimouse CD23 antibodies and magnetic depletion. In a second set of experiments, CD5$^+$ B cells were isolated by T-cell depletion, adherence, and Percoll sedimentation, followed by fluorescence-activated cell sorting (FACS) with CD5 monoclonal antibodies (MoAbs). These cells were 99.1% IgM$^+$. CD5$^-$ (B-2) B cells were isolated in a similar manner from splenocytes, except that contaminating CD5$^+$ B cells were removed by magnetic particles.

Analysis of Thy1.1 reporter expression and isolation of cells using FACS. For FACS analysis, 10^6 cells were washed in PBS and incubated with fluorescein (FITC)- or phycoerythrin (PE)-conjugated antibody to specific surface antigens for 30 minutes at 4°C in 100 μL 5% heat-inactivated fetal bovine serum/PBS. Cells were preincubated for 5 minutes with 2 μg mouse MoAb from clone 2.4G2 recognizing CD32/CD16 (FcγII/III), which blocks nonspecific binding to murine Fc receptors present on phagocytes.[24] Cells were then washed in PBS and immediately subjected to FACS analysis or

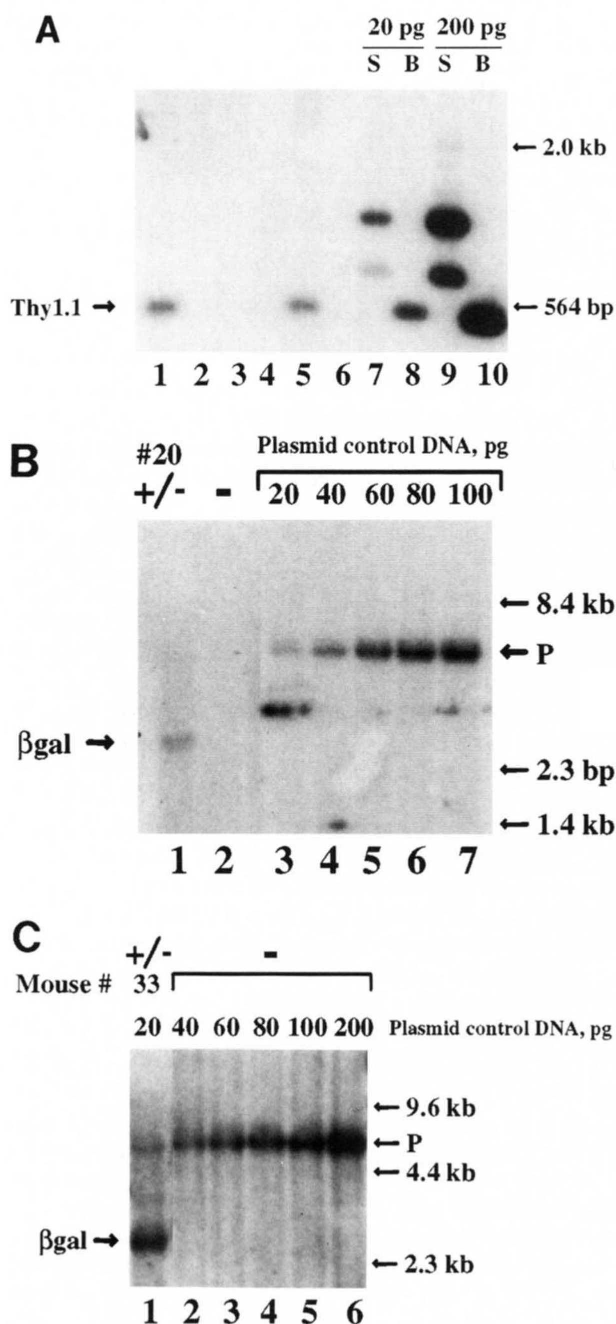

Fig 2. Southern blot analysis of CD11b transgene tail DNA. (A) Thy1.1 reporter. In lanes 1 through 6, 10 μg of genomic DNA from various progeny was digested with *Bam*HI, separated on a 1% agarose gel, and hybridized to the 544-bp Thy1.1 cDNA probe. Lanes 7 and 9 contain the CD11b/Thy1.1 transgenic plasmid (Fig 1B) digested with *Sst* I; the probe hybridizes to fragments of approximately 1,140 bp and 700 bp. Lanes 8 and 10 contain 20 and 200 pg, respectively, of the transgenic construct cut with *Bam*HI. Ten micrograms of genomic DNA from a nontransgenic animal was also included in lanes 7 to 10. The positions of the 2-kb and 564-bp λ/*Hin*dIII markers are noted on the right, and that of the 544-bp Thy1.1 insert on the left. DNA in lanes 1 and 5 are positive; lanes 2, 3, 4, and 6 are negative for containing the transgene. Exposure was for 24 hours with an intensifying screen. (B) β-gal reporter founder no. 20. Ten micrograms of genomic DNA were digested with *Sst* I and analyzed as in (A), except that the probe was a 2.0-kb *Sst* I fragment from the LacZ gene from the plasmid pSDKlacZpA (Fig 1C).[21] Lane 1 is DNA from founder no. 20, whereas lane 2 is from a nontransgenic littermate. In lanes 3 to 7, increasing amounts of the lacZ plasmid pSDKlacZpA were digested with *Bam*HI and mixed with 10 μg of genomic DNA from a nontransgenic mouse before running on the gel. Arrows on the right indicate the positions of the 8.4-kb, 2.3-kb, and 1.4-kb *Bst*EII-digested λ DNA markers. "P" indicates the position of the 6.1-kb fragment generated by linearization of the plasmid pSDKlacZpA by restriction digestion with *Bam*HI. "β-gal" on the left indicates the position of the 2.7-kb *Sst* I fragment from the transgene (Fig 1C). The blot was exposed for 27 hours with an intensifying screen. (C) β-gal reporter founder no. 33. Analysis was performed as for (B) except that in this case lane 1 contained 10 μg of founder DNA and lanes 2 to 6 contained 10 μg of nontransgenic DNA. *Bam*HI-digested pSDKlacZpA plasmid DNA was added to each lane in the amounts indicated. Arrows on the right indicate the positions of the 9.6-kb, 4.4-kb, and 2.3-kb λ/*Hin*dIII-digested DNA markers. "P" indicates the position of the 6.1-kb *Bam*HI fragment from pSDKlacZpA. "β-gal" on the left indicates the position of the 2.7-kb *Sst* I fragment from the transgene (Fig 1C). The blot was exposed for 52 hours with an intensifying screen.

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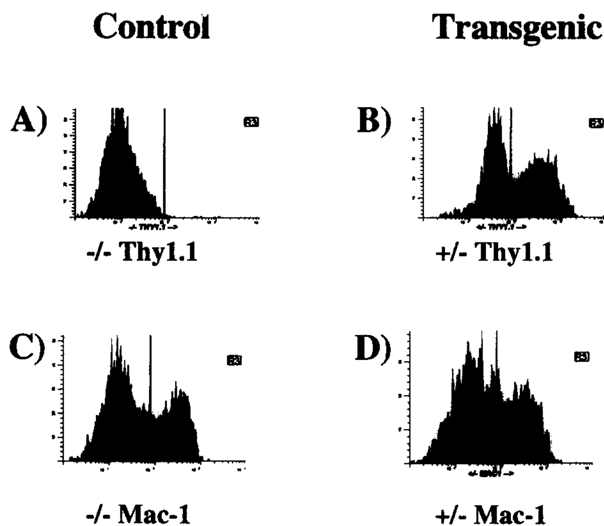

Fig 3. FACS analysis of Thy1.1 reporter expression in peritoneal macrophages. FACS analysis was performed on 72-hour thioglycollate-elicited peritoneal macrophages as described in Materials and Methods. (A) Nontransgenic littermate, Thy1.1 antibody; (B) transgenic, Thy1.1 antibody; (C) nontransgenic, mouse CD11b (Mac-1) antibody; (D) transgenic, mouse CD11b antibody. Staining of cells of both nontransgenic and transgenic animals with isotype controls was no greater than that shown in (A).

sorting on a Becton Dickinson FACStar[Plus] sorter (Becton Dickinson, Mountain View, CA). Antibodies used included Gr-1 FITC (RB6-8C5; Pharmingen, San Diego, CA) recognizing a granulocytic marker[25] that increases with maturation[26]; Mac-1 FITC (Caltag) recognizing mouse CD11b; Thy1.1 PE (Serotec; Harlan Bioproducts for Science, Indianapolis, IN), CD5 (Becton Dickinson), and B220, CD4, and CD8 (all from Caltag, South San Francisco, CA). In some experiments, dead cells were removed from analysis using 1 μg/mL propidium iodide; because greater than 95% of cells in most experiments were viable, this procedure did not significantly change any of the results obtained.

Isolation of RNA from murine tissues. Total RNA was isolated from murine tissues by homogenization in a tissue homogenizer (Brinkmann, Westbury, NY) in 4 mol/L guanidium isothiocyanate, 17 mmol/L sarkosyl, 250 mmol/L sodium citrate, pH 7.0, and 0.1% β mercaptoethanol, followed by cesium chloride centrifugation.[27] Total RNA was isolated from murine macrophages by adding the guanidium solution to the pelleted cells and vortexing as quickly as possible, omitting the tissue homogenization step.

Northern blot analysis. Ten micrograms of total RNA was fractionated on either 1.2% or 1% formaldehyde/agarose gels, as indicated in the figure legends, and transferred by capillary blotting to nylon membranes (Biotrans; ICN, Irvine, CA). Membranes were baked at 80°C for 1 hour and UV cross-linked as described above. Blots were prehybridized, hybridized, and washed as described above for Southern blots. The probes were either the 544-bp Thy1.1 cDNA (Fig 1B) or a mouse Mac-1 cDNA probe.[28] Autoradiography was performed at −80°C with an intensifying screen for the time indicated in each figure legend. Blots were then hybridized to an [α-^{32}P]-dCTP 3′ end-labeled[29] 18S oligonucleotide probe overnight in 6× SSC, 5× Denhardt's solution and 0.5% SDS at 48°C; washed twice in 2× SSC and 0.2% SDS at room temperature for 5 minutes; and washed once in 0.2× SSC and 0.2% SDS at 48°C for 30 minutes. The oligonucleotide probe for 18S rRNA was bp 938 to 921 of the human 18S ribosomal RNA gene, 5′-TCGGGCCTGCTTTGAACA-3′.[30] Intensity of bands was quantitated using Imagequant phosphorimager software (Molecular Dynamics).

X-gal staining of cells for detection of β-galactosidase activity. Peritoneal cells were washed in PBS, fixed in 0.75% formaldehyde, 0.05% glutaraldehyde, washed in PBS plus 2 mmol/L $MgCl_2$, and incubated in 7 mmol/L $K_3Fe(CN)_6$, 7 mmol/L $K_4Fe(CN)_6$, 2 mmol/L $MgCl_2$, 0.02% NP-40, and 1 mg/mL Xgal (diluted from a 2% stock in N,N-dimethylformamide [DMF]).[21] In general, the best discrimination between transgenic and nontransgenic expression was obtained after 8 to 18 hours at 37°C.

RNAse protection assay for lacZ mRNA. A plasmid containing a 290-bp fragment of the *Escherichia coli* lacZ gene, corresponding to bp 836 to 1125,[22] was cloned into the *Cla I/Eco*RV sites of the plasmid pBluescript II KS+. The plasmid was linearized with *Xho* I, and a 378-bp antisense probe labeled with α^{32}P-UTP was generated by incubation of 1 μg of linearized plasmid with 20 U T7 polymerase as described.[31] The probe was purified on 6% acrylamide gels. A total of 5×10^5 cpm (specific activity, 2.8×10^7 cpm/μg) of probe was incubated with 20 μg of total RNA for 16 hours at 45°C in 80% formamide, 400 mmol/L NaCl, 1 mmol/L EDTA. Single-stranded RNA was digested by incubation with 200 μg/mL RNAse A and 2 U RNAse T1. Products were separated on a 6% acrylamide/7 mol/L urea gel, dried, and autoradiographed. Size standards used were *Msp* II-digested pBR322 DNA fragments end-labeled using T_4 polynucleotide kinase and γ^{32}P-ATP.

RESULTS

Generation of transgenic mice harboring the −1.7-kb CD11b promoter and two different reporter genes. Previous transient transfection studies in tissue culture cell lines indicated that a 1.7-kb fragment upstream of the CD11b transcription start site[20] directed high-level expression in myeloid lines and relatively little expression in nonmyeloid lines such as HeLa.[7,12] Recent studies have indicated that this promoter also directs specific expression in human $CD34^+$ cells induced to differentiate into myeloid cells.[32] To test whether the same activity and specificity could be observed in normal myeloid cells in transgenic mice, constructs were produced in which the CD11b promoter, containing −1,704 bp of 5′ upstream sequence and 83 bp of 5′ untranslated DNA, was used to drive the expression of two different reporters (Fig 1). In the first instance, the CD11b promoter was used to drive the expression of a murine Thy1.1 cDNA as a reporter. The Thy1 gene encodes a surface glycoprotein expressed on lymphoid and neural cells.[33] This construct was introduced into mice that are homozygous for the Thy1.2 allele, which differs from Thy1.1 by a single amino acid change.[33,34] Thy1.1 and Thy1.2 can be distinguished by allele-specific MoAbs; therefore, expression of the transgene can be easily assessed by FACS analysis. In the second construct, the same −1,704- to +83-bp CD11b promoter was placed in front of the *E coli* lacZ gene, which can be easily assayed by staining for β-galactosidase activity using Xgal as a substrate. We chose this as an alternate reporter because previous investigators had shown its utility in analysis of the activity of the platelet factor 4 promoter in hematopoietic cells.[21]

These two different constructs were used to generate transgenic founder mice, which were then bred to establish stable lines. We were able to derive three founder lines heterozygous for the transgene that were analyzed in this study.

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The single Thy1.1 reporter line had an estimated copy number of 5 genome equivalents as estimated by Southern blot analysis (Fig 2A), whereas the two β-gal reporter lines had estimated copy numbers of 1 for founder no. 20 and 5 for founder no. 33, respectively (Fig 2B and C).

The CD11b promoter directs high-level expression of reporter genes in macrophages in transgenic mice. We first screened for expression of the Thy1.1 founder by measuring the surface expression of the Thy1.1 reporter in peritoneal macrophages. FACS analysis of these cells, which are greater than 90% macrophages and express high levels of murine CD11b (Mac-1) (Fig 3), demonstrated very high levels of the Thy1.1 epitope, which was not present on cells from the nontransgenic littermate, which expresses only the endogenous Thy1.2 antigen (Fig 3). To assess the relative level of RNA produced by the CD11b promoter in comparison to the endogenous murine CD11b promoter, we performed Northern blot analysis (Fig 4). Because the Thy1.1 and Thy1.2 antigens differ by only one amino acid, both RNAs are detected simultaneously in Northern blots. The transgenic Thy1.1 RNA can be distinguished from the endogenous Thy1.2 RNA, which is slightly greater in size. Although we could detect no Thy1 mRNA in peritoneal macrophages from nontransgenic littermates (Fig 4A, lane 1), a very high level of expression was observed in the CD11b/Thy1.1 animal (Fig 4A, lane 2). We compared the amount of RNA expressed in the transgene with that of the endogenous CD11b gene in macrophages (Fig 4B, lane 9) by densitometry of the signal on Northern blots and correcting for the time of exposure, intensity of the 18S signal, and copy number. We estimate by this analysis that the transgene expresses at approximately two thirds the strength of the endogenous mouse CD11b gene at the RNA level. Significant levels of the transgene RNA could also be detected in bone marrow and spleen, as well as to a lesser degree in lung (it should be noted that the blot in Fig 4A was exposed for only 3.5 hours). We were unable to detect significant levels of the transgene RNA in other tissues sampled, including kidney and thymus, even after long exposure of the blot. As expected, we observed high levels of the endogenous Thy1 RNA in brain and thymus. This pattern of transgenic RNA expression is similar to that observed with the endogenous murine CD11b gene, in which high levels are observed in macrophages and bone marrow and lesser amounts in spleen (Fig 4B).

We also observed high levels of reporter gene expression in both β-gal founder lines examined. An example of the Xgal staining observed in peritoneal macrophages from founder no. 20 is demonstrated in Fig 5. A faint blue color can be observed in peritoneal macrophages from nontransgenic littermates (Fig 5A), which presumably reflects

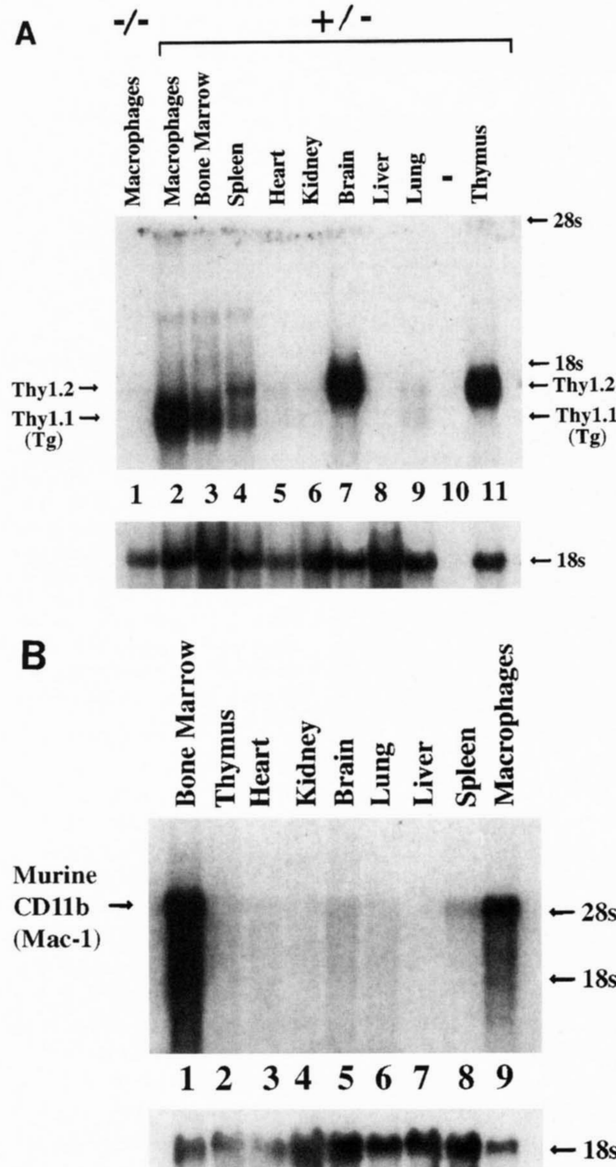

Fig 4. Northern blot analysis of Thy1.1 reporter and endogenous murine CD11b expression in various tissues. (A) Northern blot analysis of the Thy1.1 reporter in transgenic mice. Total RNA was prepared from various tissues and 10 μg was run in each lane on a 1.2% agarose formaldehyde gel before transfer and hybridization to the Thy1.1 cDNA probe (upper panel). Tissues analyzed are indicated above the blot. Lane 1 contained 10 μg of RNA from peritoneal macrophages from a nontransgenic (−/−) littermate. Lane 2 contained RNA from 72-hour thioglycollate-elicited peritoneal macrophages from the transgenic (+/−) animal. Lane 10 contained no RNA. Positions of the 28s and 18s ribosomal markers are shown on the right and of the endogenous Thy1.2 and transgenic Thy1.1 mRNAs on the left and the right. The autoradiogram was exposed for 3.5 hours with an intensifying screen. The bottom panel shows an autoradiogram after hybridization to an 18s oligonucleotide (exposed for 30 minutes with a screen). (B) Endogenous murine CD11b (Mac-1) expression. Ten micrograms of total RNA from a nontransgenic littermate was run in each lane on a 1.0% agarose formaldehyde gel before transfer and hybridization to a murine CD11b (Mac-1) cDNA[28] probe (upper panel). Tissues analyzed are indicated above the blot. Lane 9 contained 72-hour thioglycollate-elicited peritoneal macrophages. Positions of the 28s and 18s ribosomal markers are shown on the right and of the 6-kb endogenous murine CD11b mRNA on the left. The autoradiogram was exposed for 16 hours with an intensifying screen. The bottom panel shows hybridization to the 18s oligonucleotide (exposed for 30 minutes with a screen).

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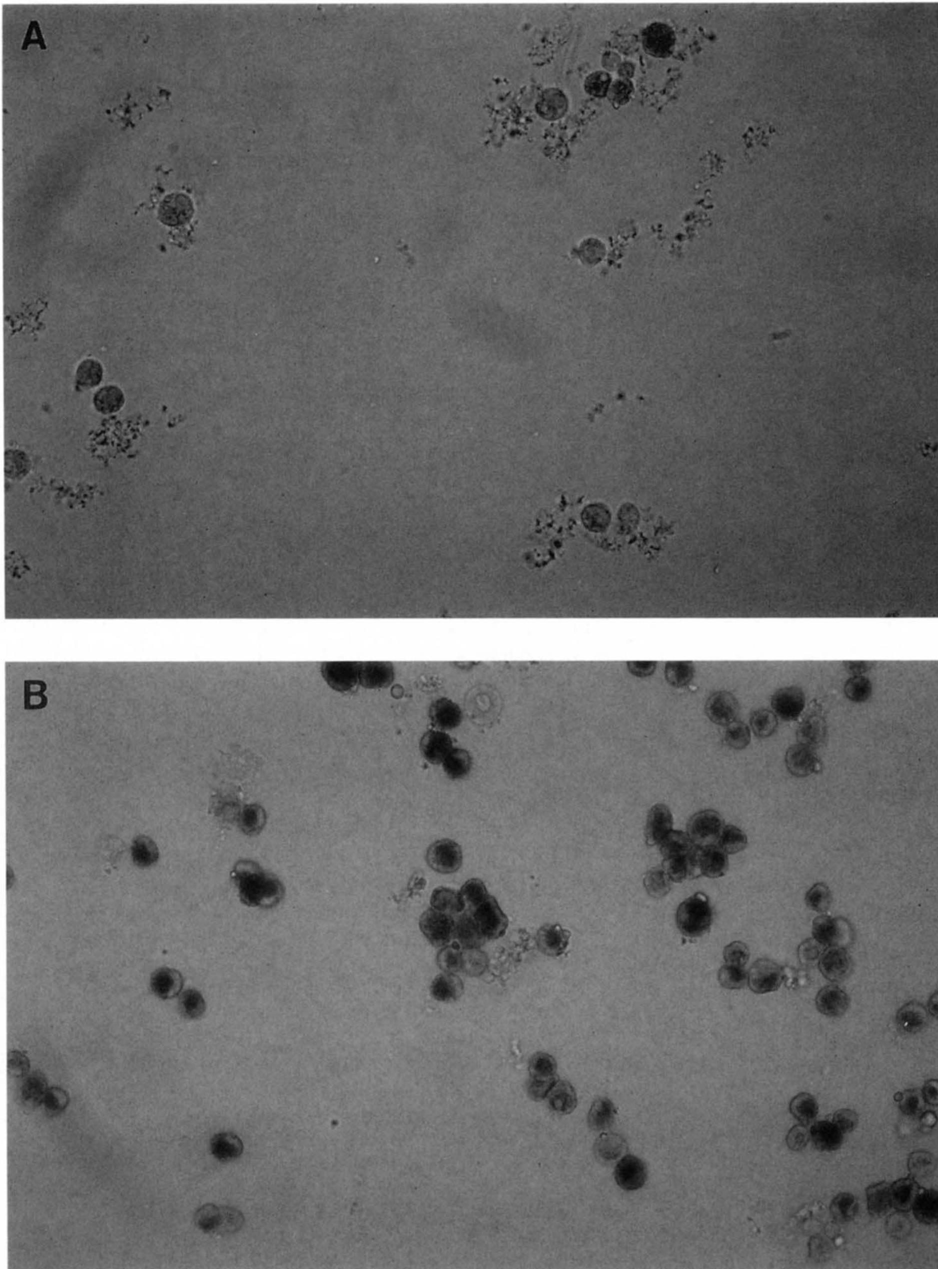

Fig 5. Analysis of β-gal reporter staining in peritoneal macrophages. (A) Nontransgenic control peritoneal macrophages. Peritoneal macrophages were elicited by administration of thioglycollate and collected by lavage 72 hours later. The cells (90% macrophages) were then incubated with Xgal and stained for 18 hours as described in Materials and Methods. (B) CD11b/β-gal transgenic peritoneal macrophages. The cells were isolated from founder no. 20 strain and treated exactly as described above.

some endogenous β-galactosidase–like activity. This color did not deepen with longer incubation times. The blue staining of the CD11b/β-gal macrophages is far more intense in color (Fig 5B). A similar increase in β-gal activity compared with that in nontransgenic macrophages was noted with founder no. 33 as well, although the intensity of staining was less than that of founder no. 20, even though founder no. 33 was estimated to harbor 5 times as many copies. Almost all (90%) of the peritoneal cells in these cultures appear to be macrophages on the basis of morphology after Wright-Giemsa staining. We also used FACS to separate these peritoneal cells into murine $CD11b^+$ ($Mac\text{-}1^+$) and $CD11b^-$ populations. All of the blue cells segregated to the mouse $CD11b^+$ ($Mac\text{-}1^+$) population; only background staining (equivalent to nontransgenic control) was observed in the mouse $CD11b^-$ cells. Finally, to confirm that the β-gal staining reflected production of significant amounts of RNA by the promoter, we performed RNAse protection analysis of β-gal RNA (Fig 6). We were able to observe high levels of β-gal RNA in CD11b/β-gal macrophages, similar to the levels observed in a line of U937 cells stably transfected with a β-gal reporter driven by a strong SV40 viral promoter (compare Fig 6, lanes 2 and 4). In summary, all three founder lines expressed significant levels of reporter RNA in macrophages. This expression was observed consistently from generation to generation and in animals that were nearly 2 years old.

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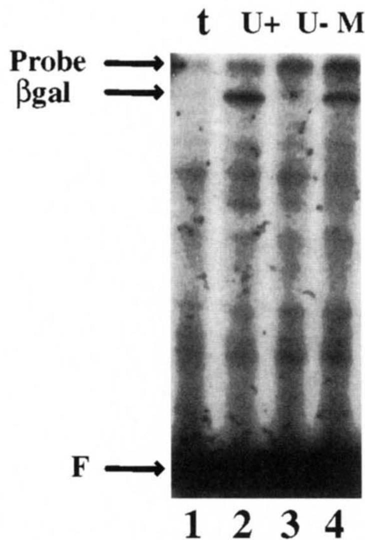

Fig 6. Analysis of β-gal reporter RNA by RNAse protection assay in peritoneal macrophages. Peritoneal macrophages isolated as described above were subjected to analysis of lacZ RNA by RNAse protection assay as described in Materials and Methods using the *Cla* I/*Eco*RV lacZ fragment shown in Fig 1C as a probe. Lane 1, tRNA; lane 2, RNA from a U937 cell line stably transfected with a construct in which the SV40 promoter is used to drive expression of β-gal; lane 3, control (untransfected) U937 cells; lane 4, peritoneal macrophages from founder no. 20. "Probe" indicates the position of the 378-bp probe. "β-gal" indicates the position of the 290-bp band specifically protected by β-gal RNA. The sizes of the probe and protected band matched that predicted by comparison with end-labeled *Msp* II-digested pBR322 DNA fragments. "F" indicates free probe. Exposure was for 68 hours with an intensifying screen.

The CD11b promoter is expressed predominantly in mature myeloid cells. We observed relatively high levels of CD11b/Thy1 reporter RNA in macrophages compared with bone marrow (Fig 4). After normalizing the intensity of the Northern blot bands to the copy number and 18s ribosomal bands, the transgene was found to express levels of RNA that were approximately two thirds of the endogenous mouse CD11b in peritoneal macrophages, but only at approximately one tenth in bone marrow (Fig 4). This finding suggested that the transgene was predominantly expressed in mature cells and not in progenitors. To further demonstrate this finding, bone marrow cells were separated by two-color FACS with antibodies recognizing the Thy1.1 reporter and murine Gr-1. The Gr-1 MoAb recognizes a member of the Ly-6 gene family and is predominantly expressed on cells of the neutrophil lineage.[25] The intensity of staining with Gr-1 can be correlated with the degree of maturation of the cells, with Gr-1^{hi} cells representing end-stage neutrophils.[26] When CD11b/Thy1.1 transgenic bone marrow cells were stained with Gr-1 and Thy1.1, many of the Gr-1$^+$ cells were negative for Thy1.1 expression (Fig 7B). Examination of these Gr-1^{hi}/Thy1.1^{lo} cells by Wright-Giemsa staining showed that the majority were metamyelocytes, whereas almost all of the double-positive Gr-1^{hi}/Thy1.1^{hi} cells were mature polymorphonuclear granulocytes. We also performed two-color FACS of bone marrow cells with Thy1.1 and murine CD11b (Mac-1) and observed a population of Mac-1^{hi}/Thy1.1^{lo} cells in the bone marrow (Fig 7D). The majority of these cells were immature monocytic or promyelocytic cells, whereas the double-positive Mac-1^{hi}/Thy1.1^{hi} population was predominantly composed of macrophages and mature neutrophils. These findings suggest that the human CD11b promoter fragment used in these studies primarily directs expression to the mature myeloid compartment.

Each of the transgenic lines analyzed expressed significant reporter RNA in murine macrophages. Initial Northern blot analysis of the CD11b/Thy1.1 line indicated that transgene RNA was expressed only in those tissues that expressed endogenous mouse CD11b (Fig 4). However, additional FACS analysis was performed in peripheral blood and lymph nodes from mice derived from this founder (Figs 8 and 9). In peripheral white blood cells, surface expression of the Thy1.1 reporter was found in approximately 90% of Mac-1$^+$ cells (Fig 8). T cells from peripheral blood (Fig 8; similar results were obtained using surface T3 as a T-cell marker), lymph nodes (Fig 9), spleen, thymus, or the peritoneal cavity were all negative for Thy1.1 expression. However, a significant fraction of the B220$^+$ B-cell population expressed the Thy1.1 reporter, both in peripheral blood and in lymph nodes (Figs 8 and 9). Further analysis showed that many of the Thy1.1$^+$ B cells were CD5$^+$ (Ly1$^+$; data not shown). In both β-gal founders, we were unable to detect expression in purified CD5$^+$ B cells by Xgal staining, perhaps as a result of decreased sensitivity of this assay in lymphoid cells[35] compared with FACS analysis, although in founder no. 33 we were able to detect β-gal RNA in spleen (data not shown). In summary, we observed significant expression of reporter gene activity in mature myeloid cells and lesser expression in a subset of B cells. Northern blot analysis (Fig 4) indicates

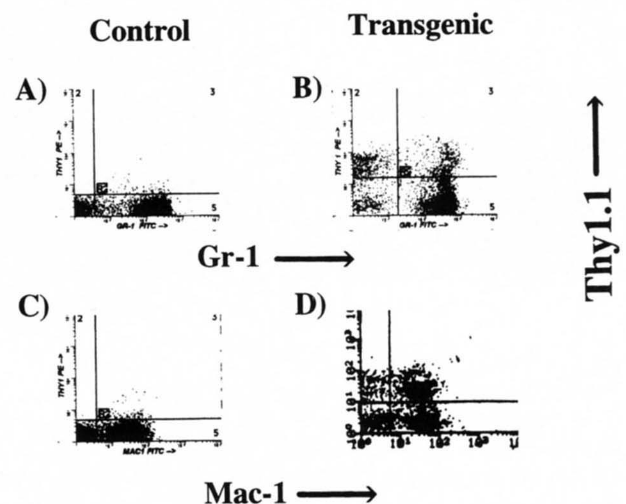

Fig 7. Two-color FACS analysis of Thy1.1 reporter expression in Gr-1$^{\pm}$ and Mac-1$^{\pm}$ bone marrow. (A and B) Staining of bone marrow with Gr-1 and Thy1.1. Bone marrow cells from nontransgenic (A) and the CD11b/Thy1.1 line (B) were isolated by flushing the long bones of the leg and subjected to FACS with Gr-1 FITC and Thy1.1 PE. (C and D) Staining of bone marrow with mouse CD11b (Mac-1) and Thy1.1. The analysis was performed as described for (A) and (B) except that mouse CD11b (Mac-1) FITC was used with Thy1.1 PE.

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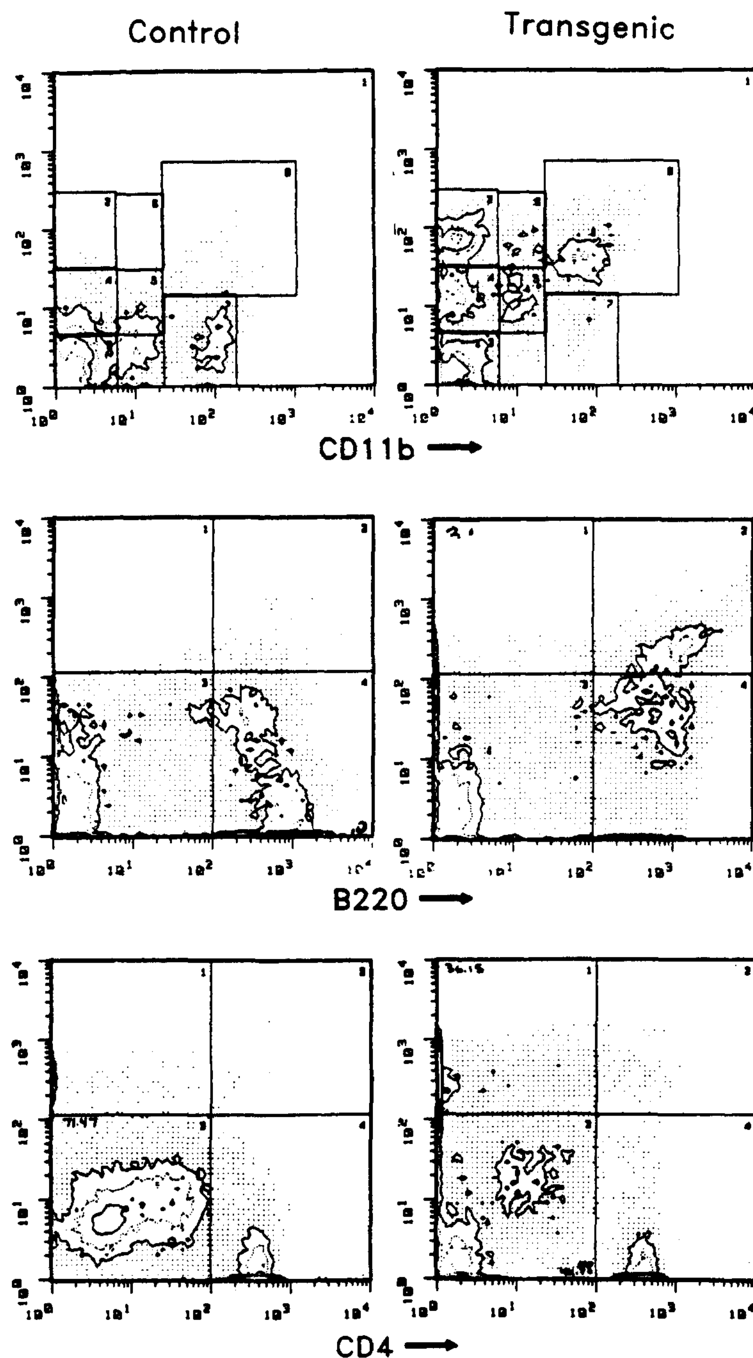

Fig 8. FACS analysis of Thy1.1 reporter expression in peripheral white blood cells. Peripheral white blood cells from control (nontransgenic) and CD11b/Thy1.1 transgenic animals and stained in each case with PE-conjugated Thy1.1 antibody (vertical axis) and FITC-conjugated antibodies recognizing either murine CD11b (top panel), the B-cell marker B220 (middle panel), or the T-cell marker CD4 (lower panel).

that the highest level of transgene RNA was expressed in peritoneal macrophages, with much lesser amounts in spleen.

DISCUSSION

In these studies, we investigated the activity of the proximal CD11b promoter in transgenic mice. Although the number of independent founders in this study was relatively small, we believe that several conclusions can be drawn from the three founder lines studied in detail. First of all, the CD11b promoter is capable of driving high-level expression of a reporter gene in myeloid cells in transgenic mice at levels comparable to those of the endogenous CD11b promoter. Secondly, the activity of the −1.7-kb promoter is highest in mature myeloid cells, both in the neutrophilic and monocytic lineages. In one founder, we observed expression in the $CD5^+$ ($Ly1^+$ or B-1) subset of B cells; previous studies have shown that this subset of B cells can express mouse or human CD11b[10,11,36]; therefore, the activity of the CD11b promoter may reflect the capability of these cells to express CD11b under certain circumstances.

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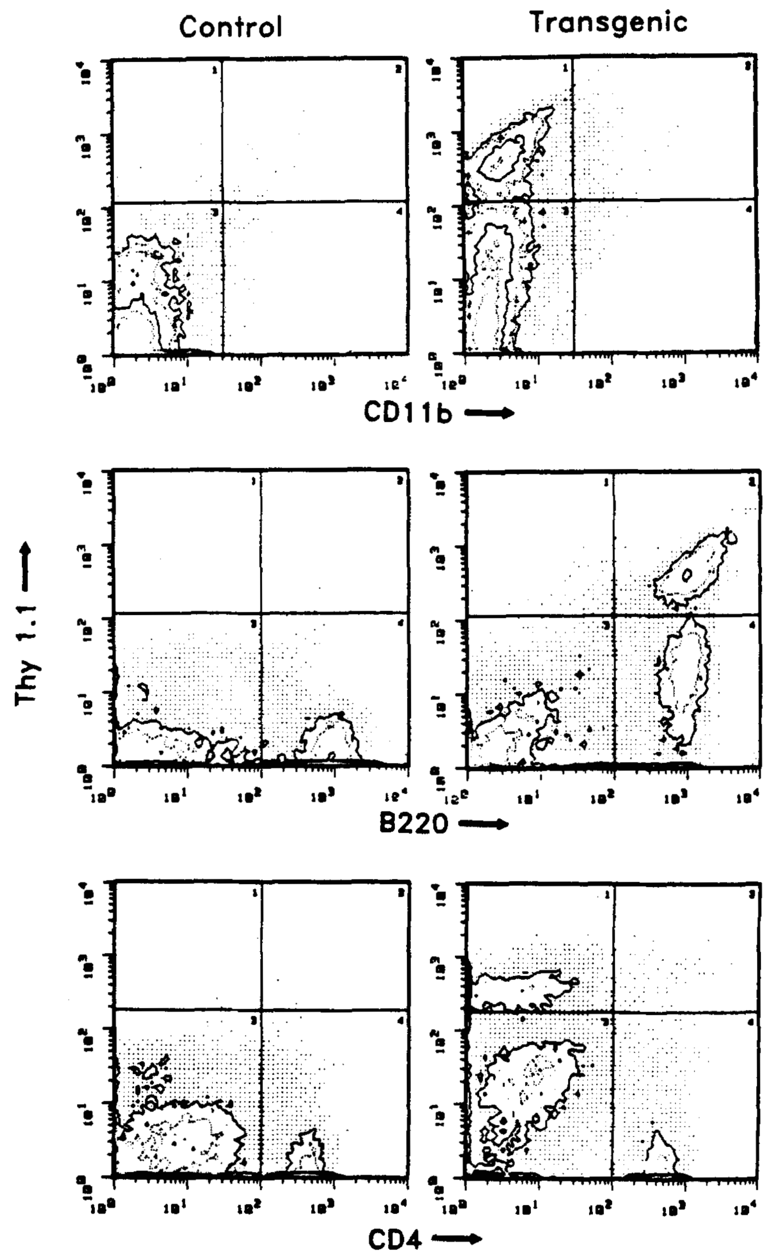

Fig 9. FACS analysis of Thy1.1 reporter expression in lymph nodes. The analysis was performed on cells obtained from lymph nodes from control (nontransgenic) and CD11b/Thy1.1 transgenic animals as described in the legend to Fig 8.

A number of transgenic studies have investigated the elements controlling myeloid gene expression, but to date none has been able to precisely define the elements required for myeloid specificity. For example, the proximal 1.5-kb of the gp91-phox gene, normally expressed at high levels in granulocytes, targeted reporter expression to a small subset of monocytic cells, but most monocytes and neutrophils did not express the transgene.[37] In the case of human c-fes/fps,[38] chicken lysozyme,[39] or human cathepsin G,[40] relatively large pieces of DNA (6 kb to 21.5 kb) were used that included the gene itself as the reporter. In each of these studies, the investigators reported high-level, properly regulated transgene expression, but the responsible regulatory elements have not yet been defined. A 5-kb portion of the monocytic monocyte colony-stimulating factor (M-CSF; CSF-1) receptor promoter was reported to target major histocompatibility complex class II I-E to macrophages,[41] and the MRP8 promoter was used to express bcl-2 in neutrophils.[42] The expression patterns of the transgenes were not described in detail in either of these studies, but were stated to be specific for macrophages and neutrophils, respectively.

CD11b, like most myeloid-specific genes, lacks a TATAA box,[7,20] and previous studies using transient transfections in tissue culture cells have shown the role of two factors, Sp1 and PU.1 (Spi-1), in the myeloid specificity of the CD11b promoter.[13,43] We are currently testing the effect of specific mutations of the binding sites for these two factors on the expression of the CD11b promoter in transgenic mice. How-

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ever, it is interesting to note that of the myeloid genes that have been shown to function in transgenic studies to date, several of them, including CD11b,[13] the M-CSF receptor,[14] and the chicken lysozyme enhancer,[17] have important functional PU.1 sites, and that several of the others, including c-fes and cathepsin G, have potential PU.1 sites in their proximal promoter regions. Therefore, we suspect that PU.1, which appears to be critical for myeloid colony formation,[19] will also be a critical factor in directing myeloid expression in transgenic studies.

Additional studies will be required to precisely define the elements required for myeloid expression of CD11b. However, our studies have shown that the −1.7-kb fragment can be used to express heterologous genes at high levels in myeloid cells and therefore should be useful in targeting heterologous genes in macrophages.

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